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Abstract: Bladder cancer is a frequently recurring disease with a very poor prognosis when progressed to invasive stages, and tumor-associated blood vessels play a crucial role in this process. In order to identify novel biomarkers associated with progression, we isolated blood vascular endothelial cells (BECs) from human invasive bladder cancers and matched normal bladder tissue, and found that tumor-associated BECs greatly upregulated the expression of insulin receptor (INSR). High expression of INSR on BECs of invasive bladder cancers was significantly associated with shorter progression-free and overall survival. Furthermore, increased expression of the INSR ligand IGF-2 in invasive bladder cancers was associated with reduced overall survival. INSR may therefore represent a novel biomarker to predict cancer progression. Mechanistically, we observed pronounced hypoxia in human bladder cancer tissue, and found a positive correlation between the expression of the hypoxia marker gene GLUT1 and vascular INSR expression, indicating that hypoxia drives INSR expression in tumor-associated blood vessels. In line with this, exposure of cultured BECs and human bladder cancer cell lines to hypoxia led to increased expression of INSR and IGF-2, respectively, and IGF-2 increased BEC migration through the activation of INSR in vitro. Taken together, we identified vascular INSR expression as a potential biomarker for progression in bladder cancer. Furthermore, our data suggest that IGF-2 / INSR mediated paracrine crosstalk between bladder cancer cells and endothelial cells which is functionally involved in tumor angiogenesis and my thus represent a new therapeutic target.

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Abstract

Bladder cancer is a frequently recurring disease with a very poor prognosis when progressed to invasive stages, and tumor–associated blood vessels play a crucial role in this process. In order to identify novel biomarkers associated with progression, we isolated blood vascular endothelial cells (BECs) from human invasive bladder cancers and matched normal bladder tissue, and found that tumor-associated BECs greatly upregulated the expression of insulin receptor (INSR). High expression of INSR on BECs of invasive bladder cancers was significantly associated with shorter progression-free and overall survival. Furthermore, increased expression of the INSR ligand IGF-2 in invasive bladder cancers was associated with reduced overall survival. INSR may therefore represent a novel biomarker to predict cancer progression. Mechanistically, we observed pronounced hypoxia in human bladder cancer tissue, and found a positive correlation between the expression of the hypoxia marker gene GLUT1 and vascular INSR expression, indicating that hypoxia drives INSR expression in tumor-associated blood vessels. In line with this, exposure of cultured BECs and human bladder cancer cell lines to hypoxia led to increased expression of INSR and IGF-2, respectively, and IGF-2 increased BEC migration through the activation of INSR *in vitro*. Taken together, we identified vascular INSR expression as a potential biomarker for progression in bladder cancer. Furthermore, our data suggest that IGF-2 / INSR mediated paracrine crosstalk between bladder cancer cells and endothelial cells which is functionally involved in tumor angiogenesis and my thus represent a new therapeutic target.

Key words: Insulin receptor, insulin-like growth factor 2, vascular endothelium, bladder cancer, angiogenesis, tumor hypoxia

Introduction

Bladder cancer ranks among the top five most commonly diagnosed cancers of males in North America [1] and occurs either as non-invasive (NIBC, pTa) or invasive bladder cancer (IBC, stages pT1-pT4 depending on the degree of invasiveness). NIBC have a good prognosis with 94% of patients surviving ≥5 years after tumor resection. However, 35% of them will experience cancer recurrence within the first year after surgery, requiring additional surgical interventions [2]. In contrast, the 5-year survival of patients with IBC is much lower: 68% for pT2 (muscle invasive), and <15% for pT3 and pT4 (invasion into perivesical tissue and to distant organs, respectively) [3], despite aggressive treatment including surgery (partial or complete cystectomy), radio- and chemotherapy [4]. The lack of suitable biomarkers that would allow to reliably predict patients likely to suffer from cancer recurrence and/or progression remains a major obstacle for a more efficient treatment of bladder cancer.

We previously reported that the tumor-associated blood vasculature plays an important role in the progression of bladder cancer and identified endocan, a transcriptional target of the VEGF-A, as a marker of tumor vessels and a mediator of angiogenesis [5]. However, targeted therapy to block VEGF-A (e.g. using bevacizumab) or its cognate receptors has shown no or only little survival benefits in bladder cancer (BC [6,7]), suggesting that other pathways and mechanisms involved in vascular remodeling are more relevant in the context of this disease. Furthermore, it is currently unknown how the microenvironment in IBC contributes to tumor

invasiveness and metastasis, but it is conceivable that the highly angiogenic vasculature is directly or indirectly involved in these processes. Therefore, we aimed to identify additional vascular proteins or pathways which could serve as prognostic markers and/or targets for future therapy. Transcriptional profiling of tumor-associated blood vascular endothelial cells (tBEC) from IBC compared to endothelial cells from adjacent normal bladder tissue (nBEC) revealed a strong upregulation of insulin receptor (INSR). Importantly, we found that vascular INSR expression levels closely correlated with survival parameters, suggesting it as a new biomarker for disease progression. Concomitantly, the INSR ligand IGF-2 was upregulated in the cancer tissue, suggesting that there is active crosstalk between cancer and endothelial cells via IGF-2/INSR signaling in IBC. In addition, we identified tissue hypoxia as the underlying mechanisms for the upregulation of INSR and IGF-2, and investigated the functional role of this pathway in angiogenesis and tumor progression both *in vitro* and in a mouse model of cancer.

Materials and methods

Tumor and normal tissue samples

Clinically annotated frozen and paraffin embedded cancer and normal tissue samples were obtained from the University Hospital Zurich (USZ). Tissue collection was approved by the SPUK-GGU-USZ Ethics Committee (KEK-StV-Nr 02/09) and written informed consent was obtained from each patient.

Laser-capture microdissection and transcriptional profiling of blood vessels from bladder tissue

Laser capture microdissection on frozen samples of IBC (5 patients; pT1-pT4), RNA isolation, cDNA generation and microarray hybridization were performed as described [5]. The microarray data are available at GEO under accession number GSE41614.

Immunohistochemistry on human paraffin embedded tissue samples

Immunohistochemical stainings of paraffin-embedded tissue sections and the TMAs were performed as described [5], using a mouse monoclonal anti-human INSR antibody (4 µg/ml, clone CT-3, AHR0271, Invitrogen, USA), using a rabbit polyclonal anti-human GLUT1 antibody (1:1000, AB1341, Merck Millipore, USA), as well as biotin-labeled horse anti-mouse (5 µg/ml). Signals were detected with avidin–coupled peroxidase and 3-amino-9-ethylcarbazole (AEC) or 3,3-diamino-benzidine (DAB).

Endothelial cell migration assays

The monolayer wound healing assay was performed as described [8]. See Supplementary materials and methods for details.

Tumor mouse model

Tie2-Cre and $INSR^{fl/fl}$ mice on the C57Bl6 background have been described previously [9-11] and were bred in house to generate $Cre^{+}INSR^{fl/fl}$ mice lacking endothelial INSR expression and $Cre^{-}INSR^{fl/fl}$ control littermates. MB49 BC line [12] was obtained from Dr. Angelica Loskog (University of Uppsala) and Dr. Martin Sommerauer (University of Luebeck). Luc2-expressing MB49 murine BC cells were cultured in DMEM (Life Technologies) with 10% FBS. Tumor cells ($1x10^5$) were

injected s.c, into the flank of female $Cre^{+}INSR^{fl/fl}$ mice (N=13) or $Cre^{-}INSR^{fl/fl}$ control littermates (N=11) mice (performed under inhalation anesthesia-2.5% isolfluorane).

The smallest and largest tumor diameter were measured with a digital caliper every second day, and tumor volumes were calculated as: volume = π/6 x (smaller diameter^2 x larger diameter). On day 14 after tumor implantation, animals were injected i.p. with the hypoxia marker pimonidazole hydrochloride (NPI) (60 mg/kg, 1 h before sacrifice) and with luciferine (150 mg/kg, 10 min before sacrifice). Right after sacrifice, the primary tumor, inguinal and axillary lymph nodes, lungs and livers were dissected, and imaged using an IVIS system (Caliper). All experiments were performed in accordance with animal protocol 12/2015 approved by the Kantonales Veterinaeramt Zurich.

Results

High expression levels of insulin receptor on blood vessels of bladder cancers are associated with shorter progression-free and overall survival of patients.

We previously performed a transcriptional analysis of bladder cancer-associated blood endothelial cells (tBEC) compared to adjacent normal bladder endothelial cells (nBEC) using laser-capture microdissection (LCM) and microarray analysis [5]. The high purity of captured endothelial cell content was confirmed by high expression of PECAM1 (endothelial cell marker; among 15% highest expressed genes) and low expression of KRT7 (urothelial cell marker; among 10% lowest expressed genes).

We noticed a highly significant upregulation (>4-fold upregulation, $p_{adj}<0.01$) of insulin receptor (INSR) in tBEC, and decided to investigate this phenomenon further, due to the potential effects of this important receptor on cell metabolism and

angiogenesis. RT-qPCR analysis confirmed that INSR mRNA expression was highly increased in 5 samples of tBECs compared to nBECs (**Figure 1A**), and the presence of INSR protein expression on tumor-associated vessels of BC specimen was validated by immunostaining (**Figure 1B**). Of note, immunohistochemical analysis revealed that INSR is upregulated in the vasculature of various other solid tumor types (**Supplementary Figure 1**). INSR expression was however undetectable on blood vessels in various healthy tissues, indicating that INSR is a general tumor vessel marker (**Supplementary Figure 2**). Semi-quantitative measurement of vascular INSR expression in 63 samples of IBC (**Figure 1C, Supplementary Table S1**) followed by Kaplan-Meier analysis revealed that the progression-free survival and the overall survival of patients with strong vascular INSR expression (n=12, N_{events}=8 and n=12, N_{events}=9, respectively) was much shorter than of those with no, or weak expression (n=51, N_{events}=19, p=0.003, **Figure 1D**; n=51, N_{events}=23, p=0.004, **Figure 1E**). We also analyzed INSR expression in 90 NIBC cases ([13], **Figure 1F; Supplementary Table S2**). The patients with strong vascular INSR expression (n=53, N_{events}=31) had a tendency for shorter recurrence-free survival times than those with no or weak expression (n=37, N_{events}=16, p=0.087; **Figure 1G**). In contrast, INSR staining in tumor cells was absent or very weak in all tumor samples we analyzed, indicating that tBECs are the strongest INSR-expressing cells within the BC microenvironment (**Figure 1C, 1F**). Using a publicly available TCGA dataset on invasive cancer [14], we observed that INSR was significantly upregulated in papillary-like Luminal Cluster I ((N (Cluster I)= 43; N (Cluster II)=50, p<0.01; N (Cluster III)=35, p<0.001; N (Cluster IV)=15, p<0.001; **Supplementary Figure 3A)**. Interestingly, the same cluster showed significant upregulation of endocan, a molecule that we have previously identified to correlate with invasiveness of BC and

angiogenesis [5]. Moreover, the same cluster showed significant downregulation of the thromspondin-1 (TSP1). We have previously found that TSP1 is anti-angiogenic and has tumor-suppressing activity [15,16]. Besides, IBC pateints with low TSP1 expression showed decreased overall survival and increased microvascular density [17]. Based on these findings, it is possible that the papillary-like Luminal Cluster I is particularly dependent on angiogenesis and could benefit from anti-angiogenic therapy.

We also compared the expression of INSR in IBC of different histology from the TCGA database [14] and identified a significantly higher expression ($p<0.001$) in papillary (N=137) vs. non-papillary BC (N=285, **Supplementary Figure 3B**). BCs with lymphovascular invasion (N=88) had a significantly upregulated INSR expression ($p<0.01$, **Supplementary Figure 3C**) compared to tumors without signs of lymphovascular invasion (N=160). Moreover, BCs that metastasized (N=7) showed significantly increased levels of INSR ($p<0.05$, **Supplementary Figure 3D**) versus BCs without any evidence of metastasis (N=69).

INSR expression by blood vascular endothelial cells is upregulated upon hypoxia

Due to their high rate of proliferation and chaotic blood vessel network, the majority of cancers are hypoxic [18,19]. As we saw high expression of INSR on tBEC in majority of solid cancer, we therefore hypothesized that hypoxia might be responsible for endothelial INSR expression. Human umbilical vein endothelial cells (HUVECs) and human dermal blood vascular endothelial cells (BECs) were exposed to hypoxia for 24h, and the expression of INSR was analyzed by RT-qPCR. Hypoxia led to increased INSR expression (**Figure 2A**; 1.7-3.1-fold and 1.4-3.8-fold, respectively). On the other hand, among four BC cell lines tested, only the one

derived from high-grade cancer (T24; grade III) upregulated INSR expression upon hypoxia (1.4-2.2-fold; **Figure 2B**). This indicates that INSR expression is regulated by hypoxia specifically in endothelial cells.

In order to test whether hypoxia might also be responsible for vascular INSR expression in human BC, we indirectly assessed the level of hypoxia in 63 IBC cases (same as in **Figure 1C, Supplementary Table S1**) by staining for the hypoxia-responsive marker GLUT1 (SLC2A1; **Figure 2C**, [20,21]). Semi-quantitative scoring of GLUT1 expression revealed a clear trend for association between INSR and GLUT1 staining (GLUT1 low/INSR low=27, GLUT1 high/INSR low=24, GLUT1 low/INSR high=3, GLUT1 high/INSR high=9 p=0.08, **Figure 2D**). Of note, significantly increased expression of GLUT1 was also detected in LCM-isolated blood vascular endothelial cells from 5 BCs (tBEC) compared to those from the adjacent normal bladder tissue (nBEC), suggesting that the tBECs themselves were also hypoxic to some extent (516.5–6897.0-fold; **Figure 2E**). Furthermore, GLUT1 expression and INSR expression were significantly correlated in these samples (p=0.02, r=0.72, **Figure 2F**). In line with this, in addition to high intensity staining of GLUT1 in tumor cells, we could observe a weak GLUT1 staining in vessels in the vicinity of tumor cells. Furthermore, GLUT1 upregulation was also detectable in BECs exposed to hypoxia *in vitro* (4.4-6.0-fold; **Supplementary Figure 4A**).

GLUT1 has been shown to be induced by the hypoxia-inducible factor 1A (HIF1A, [22]), a transcription factor that becomes transcriptionally active upon a decrease in tissue oxygen levels, and that induces the expression of several hypoxia response genes [23]. We therefore hypothesized that INSR is also under control of HIF1A in endothelial cells. Using publically available ChIP-Seq data from the ENCODE project (see Supplementary materials and methods for details), we assessed whether the

hypoxia-driven upregulation of INSR might be directly regulated by HIF1A. Whereas we found no evidence of HIF1A binding to the promoter, we observed a HIF1A binding peak around 100 kb downstream of the transcriptional start site (TSS) in an intronic region of the INSR gene which contained all the histone signatures (enrichment of H3K4me1 and H3K27ac) typical of an enhancer element (**Figure 2F**). Interestingly, the H3K27ac mark in this putative enhancer region was only observed under hypoxia. Typically, enhancer elements interact with the TSS of a gene to up-regulate its expression. To investigate this link, we utilized ChIA-PET data [24], which map regions that are in close chromosomal proximity, and observed a clear interaction between the putative enhancer and the TSS of INSR (**Figure 2F**). Taken together, these findings suggest that the enhancer and its interaction with HIF1A and the TSS of INSR are important for the up-regulation of INSR transcription.

Insulin receptor isoform-A is the major isoform expressed in tumor-associated endothelial cells and in endothelial cells *in vitro*.

Two alternative splice isoforms of INSR have been described. The INSR-A isoform lacks exon 11 which encodes 12 amino acid residues at the C-terminus. INSR-A binds to IGF-2 with very high affinity, in contrast to INSR-B [25], that is expressed predominantly in insulin responsive tissues (liver, muscle, adipose tissue and kidney).

In LCM isolated tBEC, the majority of the INSR transcript consisted of INSR-A (**Figure 3A**), while in LCM isolated endothelial cells from healthy bladder tissue expression of both isoforms was very low (at the limit of detection for RT-qPCR; data not shown). Similarly, *in vitro* cultured BECs and HUVECs showed a higher

expression of INSR-A compared to INSR-B (14.5–31.7-fold, BECs; 6.1–24.8-fold, HUVEC).

IGF-2 is upregulated by hypoxia and higher levels of IGF-2 are associated with shorter overall survival of patients with IBC.

We then investigated if hypoxia might also induce the expression of two physiological ligands of INSR, IGF-1 and IGF-2. When subjected to hypoxia, BECs expressed increased levels of IGF-2 (1.4-1.9-fold; **Figure 4A**) but not of IGF-1 (0.9-1.1-fold; **Supplementary Figure 4B**). Cultured BC and urothelial cells reacted with an even more pronounced increase in IGF-2 expression (**Figure 4B**), while IGF-1 expression was undetectable (data not shown). To test whether hypoxia observed in human BC was associated with increased IGF-2 expression as well, we analyzed several publicly available gene expression datasets of total BC and control bladder tissue [26-28]. Analysis of these 3 datasets revealed significantly higher IGF-2 expression levels in IBC compared to normal bladder tissue (NB) and to normal bladder tissue that was adjacent to BC (NAB) (from [26]: 2.6-fold higher in IBC (n=85) than in NAB (n=45), **Figure 4C**; from [27]: 6.8-fold and 5.8-fold higher in IBC (n=13) than in NB (n=9) and NAB (n=5), respectively, **Figure 4D**; from [28]: 2.8-fold and 2.5-fold higher in IBC (n=141) than in NB (n=10) and NAB (n=58), respectively, **Figure 4E)**. Two of the datasets also included expression from NIBC, in which IGF-2 expression was also elevated (from [27]: 8.0-fold and 6.9-fold higher expression in NIBC (n=15) compared to NB (n=9) and NAB (n=5), respectively, **Figure 4C;** and from [28]: 1.8-fold and 1.6-fold higher expression in NIBC (n=24) compared to NB (n=10) and NAB (n=58), respectively, **Figure 4D)**.

The large number of IBC samples and long clinical follow-up in one of the datasets [28] allowed us to compare overall-survival of patients with higher versus lower IGF-2 expression. Based on receiver operating characteristic analysis (ROC), patients were stratified into two groups of high (log_2 expression>9.86 (n=73, N_{events}=21)) and low IGF-2 expression (log_2 expression<9.86 (n=68, N_{events}=10)). Kaplan-Meier analysis revealed that the survival times of patients with high expression of IGF-2 tended to be shorter (p=0.096, **Figure 4F)** In addition, when we analyzed the dataset from the cancer genome atlas project (TCGA) for BC [14], again dividing patients into a high and low IGF-2 expression group by ROC analysis (log_2 expression > or < 6.9 (n=86, N_{events}=31 and n=91, N_{events}=24, respectively), high IGF-2 expression was significantly associated with a shorter survival time (p<0.05, **Figure 4G**). Taken together, these data suggest that hypoxia initiates crosstalk between IGF-2 expressing BC cells and INSR expressing endothelial cells, which strongly correlates with a poor prognosis.

IGF-2 activates endothelial migration though INSR independently of VEGFR-2

Next, we sought to determine whether INSR stimulation is involved in cellular processes that contribute to angiogenesis. Using the endothelial scratch closure assay, we tested the effect of known agonists of INSR (Insulin, IGF-1, IGF-2) on endothelial migration. The strongest inducer of EC migration was IGF-2 (500 ng/ml, a concentration within the physiological range of 200-800 ng/ml [29]; 19.5-27.9% scratch closure above control, **Figure 5A**), followed by insulin (1 µM, 16.6-16.9%, **Supplementary Figure S5A**) and IGF-1 (500 ng/ml, 12.6-19.2%, **Supplementary Figure S5B**). IGF-2 used at 100 ng/ml induced cell migration almost efficiently as VEGF-A (20 ng/ml), the most powerful inducer of EC migration (11.0-23.1% relative

scratch closure above control, **Figure 5B**). The strong effect of IGF-2 compared to insulin or IGF-1 is in accordance with the predominant expression of INSR-A by cultured BECs and HUVECs noted earlier, which binds IGF-2 with high affinity. One of the ways that resistance to VEGF-A angiogenesis inhibitors arises is due to upregulation of other pro-angiogenesis factors [30,31]. Interestingly, we found that the migration-stimulatory effect of IGF-2 on cultured endothelial cells was independent of VEGFR-2 (**Figure 5B**). To confirm that the prominent activity of IGF-2 on endothelial was indeed mediated by INSR, we used an INSR-specific blocking antibody (IR Ab, [32]), an inhibitor of IGF-1R activation, picropodophyllin (PPP), and an inhibitor of both, INSR and IGF-1R activation, GSK1838705. IR Ab used at 10 nM completely blocked the IGF-2-induced migration (**Figure 5C**), PPP (50 nM) only partially inhibited the IGF-2-induced migration (**Figure 5D**) and GSK1838705 (100 nM) completely blocked the migration of IGF-2 (**Figure 5E**). Futhermore, we found that IR Ab (10 nM) blocked the effect of insulin (**Supplementary Figure S5C**) but not of IGF-1 (**Supplementary Figure S5D**) while PPP (50 nM) successfully blocked IGF-1 action (**Supplementary Figure S5E).** GSK1838705 (100 nM) blocked successfully the action of insulin and IGF-1 (**Supplementary Figure S5F-G**), as expected. Thus, BEC migration induced by insulin and IGF-2 appears to involve the INSR, whereas the effect of IGF-1 is only mediated by the IGF-1R. On the other hand, proliferation of BECs and HUVECs under normoxic and hypoxic conditions was hardly affected by treatment with insulin, IGF-1 or IGF-2 (**Supplementary Figure S6**), whereas VEGF-A (used as a positive control) induced BEC and HUVEC proliferation 1.5-2.0-fold.

Deletion of insulin receptor expression in blood vascular endothelial cells *in vivo* does not affect angiogenesis but attenuates tumor metastasis.

Finally, we aimed to assess the role of endothelial INSR expression in tumor growth, metastasis and angiogenesis, using a syngenic murine BC model (MB49). Tumor cells were injected subcutaneously into mice with an endothelial cell–specific inactivation of the INSR gene (Tie2-Cre^{+} INSR$^{fl/fl}$) and control littermates (Tie2-Cre^{-} INSR$^{fl/fl}$; **Figure 6A**). In these mice, Tie2-promoter driven Cre expression lead to an efficient deletion of INSR (**Figure 6B**). 14 days after tumor inoculation, the tumor sizes and tumor weight in the two groups were similar (**Figure 6C-D**). However, we observed a tendency towards a reduced incidence of lymph node metastasis in endothelial INSR-deficient (Cre+) mice (**Figure 6E**). No difference in the incidence in neither liver nor lung metastasis was detectable (**Figure 6F**), but we noted a tendency for a reduced size of metastasis at these sites in Cre+ mice, based on bioluminescence measurements (**Figure 6G**). Furthermore, histologic analysis of the primary tumors revealed no obvious differences in the blood vessel density (**Figure 6H**), and area, nor in the hypoxic (pimonidazole positive) area (**Figure 6H-J**), indicating that tumor angiogenesis is not dependent on INSR signaling in endothelial cells, but that endothelial INSR may be involved in the metastatic process.

Discussion

IBC is a dreadful disease, which, despite aggressive therapy such as complete cystectomy, has a high tendency to recur and a generally very poor prognosis. Our study reveals that high vascular INSR expression correlates with a much lower progression-free and overall survival in IBC. INSR may therefore represent a novel prognostic biomarker for this disease. Although associated with a much better

prognosis than IBC, NIBCs also have a high-risk to recur, and there are no biomarkers to predict recurrence [33]. Currently, patients have to undergo cystoscopies repeatedly [34], which are time-consuming, expensive and can cause damage to the urethra or lead to urinary tract infection [35]. Also in this case, high INSR expression on the vasculature of NIBC was associated with a tendency for more rapid recurrence of the tumor. Therefore, assessing INSR expression in tumor biopsies could contribute to a more secure prediction of recurrence in NIBC. Due to the limited sample size of our cohort, we were not able to reliably calculate whether vascular INSR was an independent predictor in a multivariate analysis. Therefore, additional studies using larger patient cohorts are warranted. Interestingly, in published expression datasets for IBC (TCGA, [14]) we found increased expression of INSR in the papillary-like Luminal Cluster I compared to other identified clusters. This cluster also showed upregulation of endocan and downregulation of thrombospondin 1, suggesting that this cluster could be angiogenic. Furthermore, increased expression of INSR was found in IBC of papillary histology versus IBC of non-papillary histology.

Regarding the mechanism behind INSR upregulation, our data suggest that INSR is directly triggered by hypoxia, which was frequently present in BC tissue, via activation of HIF1A. INSR expression correlated with the expression of *GLUT1*, a hypoxia responsive gene, in BC tissue, and GLUT1 expression in isolated tBEC suggested that the tumor-associated endothelium might be hypoxic itself. We also identified a putative enhancer of INSR which interacts with HIF1A and which may be responsible for the hypoxia-inducible INSR upregulation in BECs. Interestingly, we could not detect an upregulation of INSR in BC cells by immunohistochemical staining in spite of the presence of hypoxic (GLUT1-positive) regions in BC tissue,

indicating that HIF1A induces *INSR* transcription only in ECs, possibly due to an inactivation of the responsible enhancer region in BC cells.

On the other hand, hypoxia led to increased production of IGF-2 by cultured BECs and BC cells. Re-analysis of several large transcriptional profiling studies showed that IGF-2 is upregulated in human BCs compared to normal bladder tissue and that high IGF-2 expression levels were associated with reduced overall survival of patients with IBC, even though the difference was not as pronounced as for high INSR expression. The finding of elevated IGF-2 production by the tumors and preferential upregulation of INSR-A expression on the tBECs strongly suggests the activation of a paracrine cross-talk between tumor cells and tBECs, which correlates with poor prognosis.

It is interesting to note that several epidemiological studies have revealed that patients with diabetes mellitus type 2 have an increased incidence and mortality of BC [36] and a higher incidence of IBC [37]. In patients with non-muscle IBC (pTa, pT1), diabetes mellitus type 2 was reported to be an independent risk factor for recurrence and to correlate with more aggressive features of the cancer [38,39]. It can be speculated that in diabetes mellitus type 2, INSR expression levels on blood vessels are elevated to compensate for reduced insulin signaling, and that this leads to the worsened prognosis for BC in diabetes mellitus type 2 patients by the mechanisms shown in the present study. Interestingly, in our study only in the samples with INSR positive staining we could also find patients with diabetes mellitus.

Despite the prognostic value of INSR and IGF-2 expression in BC, it is not entirely clear what the functional consequence of this signaling pathway are, and whether it could serve as a therapeutic target as well. Our *in vitro* data suggest that IGF-2 binding to endothelial cell expressed INSR stimulates endothelial migration,

indicating a pro-angiogenic activity. On the other hand, we found no effect of IGF-2 on endothelial proliferation *in vitro*, and the blood vessel density in tumors of mice lacking INSR expression in endothelial cells was not altered. Therefore, IGF-2/INSR signaling may promote angiogenesis, but is clearly not required *in vivo*, at least as long as other angiogenic pathways in the tumor microenvironment remain intact. It is tempting to speculate that role of the IGF-2/INSR pathway in tumor angiogenesis is redundant with other pathways, such as the VEGF-A/VEGFR-2 pathway, and may compensate in the case of VEGF-A targeted therapy, which may explain the resistance of BC against this therapy. Indeed, our *in vitro* data shows that IGF-2 can stimulate endothelial migration even in the presence of VEGFR-2 blocking antibodies.

On the other hand, our findings that INSR deletion in endothelial cells slightly reduced tumor metastasis in tumor draining and non-draining lymph nodes, as well as in the lung and the liver, opens the possibility that INSR signaling may control other endothelial parameters, e.g. endothelial permeability, which may facilitate the systemic spread of cancer cells via the blood circulation. It is of interest that - in the TCGA expression dataset of IBC [14] - cancers with lymphovascular invasion and IBCs that metastasized had significantly increased levels of INSR.

From a pharmacological point of view, specific targeting of INSR-A, in contrast to INSR-B which is involved in the metabolic effects of insulin in muscle, adipose tissue and liver [40], could well become a novel and safe strategy in BC treatment as it avoids metabolic adverse effects. Another approach could be to target IGF-2. A dual IGF-1/-2 neutralizing antibody was indeed successful in preclinical tumor mouse studies [41]. Currently, this antibody is in clinical trials for metastatic

breast cancer [42]. Based on our results presented here, IBC may represent another indication for this drug in the future.

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Author Contributions:

FR, LCD, CP and LB performed experiments, collected and analyzed data. CP, LB and PW contributed human clinical material and annotated patient samples. FR and DT performed bioinformatics analysis of genomics data. CHH and PC performed experiments. FR, LCD, VIO and MD designed the experiments and wrote the manuscript. All authors have read the manuscript and had final approval of the submitted manuscript.

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*References 45-61 are cited in supplementary material only

Figure Legends

Figure 1. **High expression levels of INSR on blood vessels of bladder cancer are associated with shorter progression-free and overall survival of patients.** (A) Blood vessel endothelial cells (BEC) were isolated from frozen sections of invasive bladder cancers (tBEC) and adjacent normal bladder tissue (nBEC) (n=5). Insulin receptor mRNA was quantified by RT-qPCR and normalized to the mean expression in nBEC. (B) Paraffin sections of bladder cancer and normal bladder tissue were stained for insulin receptor (upper lane, dark red) and counterstained with hematoxylin. To identify the blood vessels, von Willebrand factor (vWF) was detected on the same section by immunofluorescence staining (lower lane, red). Hoechst 33342 was used as nuclear counterstain. (C) A tissue microarray of 63 invasive bladder cancers (pTa-CIS, pT1, pT2, pT3, pT4) was stained for INSR. The levels of expression on blood vessels (indicated by black arrows) were semi-quantitatively assessed by grading the intensity of the staining as absent (0), weak (1+), or strong (2+). The Kaplan-Meier curves of progression-free (D) and overall survival (E) of the patients with strong insulin receptor expression (2+) and of those with weak or absent insulin receptor expression (1+, 0) were compared. (F) A tissue microarray of 90 non-invasive bladder cancers (pTa) was stained for INSR. Insulin receptor expression on blood vessels (indicated by black arrows) was semi-quantitatively assessed by grading the intensity of the staining as absent (0), weak (1+), or strong (2+). (G) The Kaplan-Meier curves of recurrence-free survival of the patients with strong insulin receptor expression (2+) and those with weak or absent insulin receptor expression (1+, 0) were compared. Scale bars correspond to 100 μm.

Figure 2. **INSR expression by blood vascular endothelial cells is upregulated by hypoxia.**

(A) HUVECs and BECs were subjected to hypoxia (1% O_2) for 24 h. INSR mRNA expression was quantified by RT-qPCR and normalized to the mean expression by cells cultured under normoxic conditions (control). (B) INSR expression was quantified in primary human bladder cancer cell lines representing high (T24) or medium grade bladder cancer (HTB2 and HTB9), and differentiated urothelium (UROtsa), after 24 h of hypoxia (1% O_2). (C) A tissue microarray of 63 invasive bladder cancers (pTa-CIS, pT1, pT2, pT3, pT4) previously stained for INSR was stained for GLUT1. Representative examples of consecutive sections (upper panels: INSR; lower panels: GLUT1). Black arrows indicate blood vessels. Scale bars correspond to 100 μm. (D) Scoring of INSR staining for vessels and GLUT1 staining for tumors in the TMA of 63 invasive cancers. (E) As a measure for hypoxia in the tumor-associated blood vessels GLUT1 mRNA was measured by RT-qPCR in tBEC and nBEC isolated by LCM from invasive cancers of 5 patients with bladder cancer. Data are presented as mean ± SD. *P<0.05, **P<0.01. (F) Correlation of relative (delta Ct, comparison to the reference gene RPLP0) expression of INSR and GLUT1 in tBEC and nBEC from 5 patients with bladder cancer. (G) A UCSC Genome Browser screenshot of the genomic region harboring the insulin receptor gene (INSR, chr19: 7,112,266 – 7,294,011), represented by the RefSeq gene model is shown. From top to bottom, the tracks show: DNase I hypersensitivity sites from 125 cell types investigated in the ENCODE project, ENCODE ChIP-Seq chromatin signal of H3K4me1, H3K4me3, and H3K27ac from HUVEC cultured under normoxic conditions, a ChIA-PET interaction between a putative enhancer region and the transcription start site (TSS) of INSR in HUVEC cultured under normoxic conditions (from Papantonis et al., [24]), CHIP-Seq peaks of H3K27ac in HUVEC cultured under normoxic and hypoxic conditions (from Inoue et al., [43]), a ChIP-Seq peak of

HIF1A in HUVEC cultured under hypoxic conditions and absence of this peak under normoxic conditions (from Mimura et al., [44]). The red rectangles highlight the putative enhancer region and the transcriptional start site (TSS) of insulin receptor.

Figure 3. **INSR isoform-A is the major isoform expressed in tumor-associated endothelial cells and in endothelial cells *in vitro*.**

The alternatively spliced isoforms of insulin receptor, INSR–A and INSR–B were quantified by RT-qPCR in BECs isolated from frozen sections of human invasive bladder cancer (n=5, tBEC) and adjacent normal bladder tissue (n=5, nBEC) (A), as well as in cultured BECs and HUVECs (B). Data are presented as mean fold-change ± SD. *P<0.05, **P<0.01, ***P<0.001.

Figure 4. **IGF-2 is upregulated by hypoxia and higher levels of IGF-2 are associated with shorter overall survival of patients with IBC.**

(A) Expression of IGF-2 by BEC subjected to hypoxia (1% O_2) for 24 h was quantified by RT-qPCR and normalized to the mean expression by cells kept under normoxic conditions (control). (B) IGF-2 expression was quantified in primary human bladder cancer cell lines representing high (T24) or medium grade bladder cancer (HTB2 and HTB9), and differentiated urothelium (UROtsa), after 24 h of hypoxia (1% O_2). (C-E) IGF-2 expression in both invasive and non-invasive bladder cancers (IBC and NIBC) was compared to IGF-2 expression in normal bladder tissue (NB) and/or normal tissue adjacent to bladder cancers (NAB) using the publicly available datasets from Sanchez-Carbayo et al. ([26], C), Dyrskjot et al. ([27], D), and Lee at al. ([28], E). (F-G) Kaplan-Meier curves of patient survival were compared between the IBCs expressing higher IGF-2 levels and those expressing lower levels in the study by

Lee et al. (F) and between the bladder cancers expressing higher and lower IGF-2 levels from the TCGA (G). Data are presented as mean ± SD. *P<0.05, **P<0.01, ***P<0.001. Horizontal lines in (C-E) denote mean values.

Figure 5. **IGF-2 is potent activator of endothelial migration through INSR, independently of VEGFR-2 inhibition.**

Migration of BECs in response to increasing concentrations of (A) IGF-2 was assessed using a scratch closure assay. Significance testing was performed between control and treated samples. (B) Migration induced by VEGFA and / or IGF-2 was measured in presence of a VEGFR-2 blocking antibody or an isotype-matched irrelevant antibody. (C-E) Increasing concentrations of an INSR blocking antibody (IR Ab, C), IGF-1R inhibitor (PPP, D) and dual INSR and IGF1R inhibitor (GSK1838705, E) were used to assess the contribution of INSR and IGF-1R in IGR-2 induced migration. Data from one representative experiment are presented as mean ± SD. *P<0.05, **P<0.01, ***P<0.001.

Figure 6. **Loss of insulin receptor expression on blood vascular endothelial cells *in vivo* attenuates bladder cancer metastasis.**

(A) Schematic diagram of the transgenic mouse genomic constructs used to generate C57Bl6 mice lacking INSR on BECs (Tie2-Cre^{+} INSR$^{fl/fl}$). (B) RT-qPCR quantification on the extent of exon 4 deletion from the INSR locus in FACS sorted endothelial cells from the ear dermis. (C) The volume of MB49 tumors injected s.c. in control (Tie2-Cre^{-} INSR$^{fl/fl}$ (Cre-); n=11) and in mice lacking INSR on blood vascular endothelial cells (Tie2-Cre^{+} INSR$^{fl/fl}$ (Cre+); n=13) was measured every second day. (D) Tumor weight was measured on the day of sacrifice. (E) Lymph

nodes (LN) from tumor-bearing Cre+ or Cre- mice were assessed for incidence of metastasis using bioluminescence imaging. (F) Lung and liver from tumor-bearing Cre+ or Cre- mice were assessed for incidence of metastasis using bioluminescence imaging. (G) Using the bioluminescence signal intensity, the extent of metastasis in Cre+ and Cre- mice was quantified. (H-I) MB49 tumor sections from Cre+ and Cre- mice were stained for MECA32 (mouse blood vascular endothelial cell marker) and number of vessels (H) and area covered by vessels (I) was assessed. (J) Tumor hypoxia was analyzed by staining for pimonidazole-protein adducts. Data are presented as mean ± SD.

SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods YES

Supplementary figure legends YES

Figure S1. Insulin receptor is highly expressed on blood vessels in a variety of human solid tumors

Figure S2. Insulin receptor is not expressed on blood vessels of healthy human tissues

Figure S3. Insulin receptor expression is elevated in invasive bladder cancers with molecular profiles of the papillary-like luminal cluster I and in bladder cancers with papillary histology, and correlates with lymphatic invasion and metastasis

Figure S4. GLUT1 is upregulated by hypoxia in endothelial cells *in vitro* while IGF-1 is not

Figure S5. Insulin receptor mediates the stimulation of human blood vascular endothelial cell migration by insulin while IGF-1R mediates the effects of IGF-1

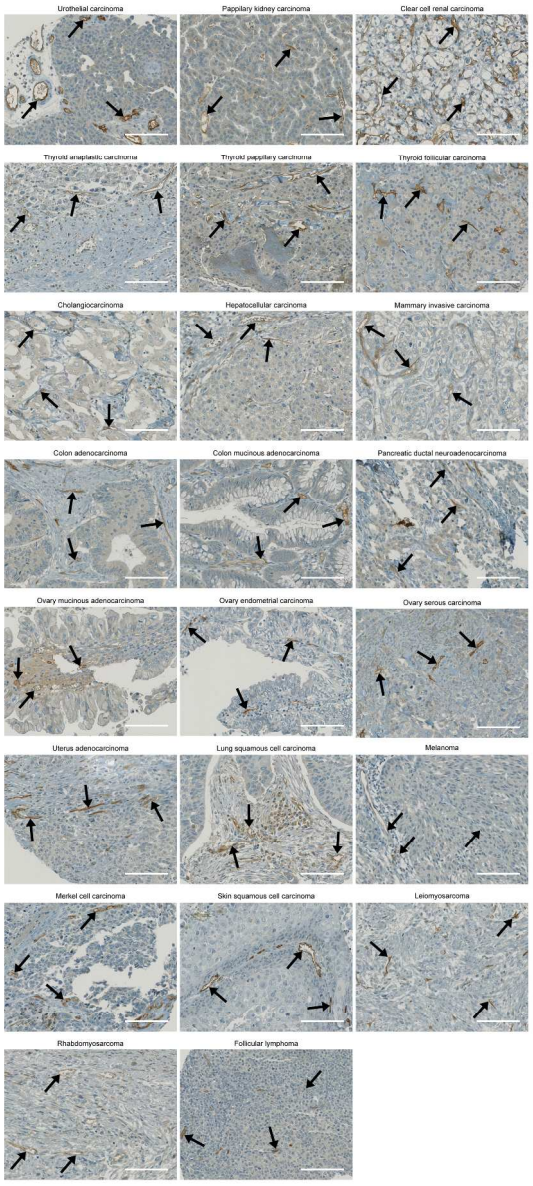
Figure S6. Insulin, IGF-1 and IGF-2 have no major effect on the proliferation of cultured blood vascular endothelial cells under normoxic or hypoxic conditions

Table S1. Clinico-pathologic characteristics of the patients with invasive bladder cancer whose samples are contained in the TMA. Quantification of immunohistochemical staining for insulin receptor on blood vessels and GLUT1 on tumor cells in the TMA

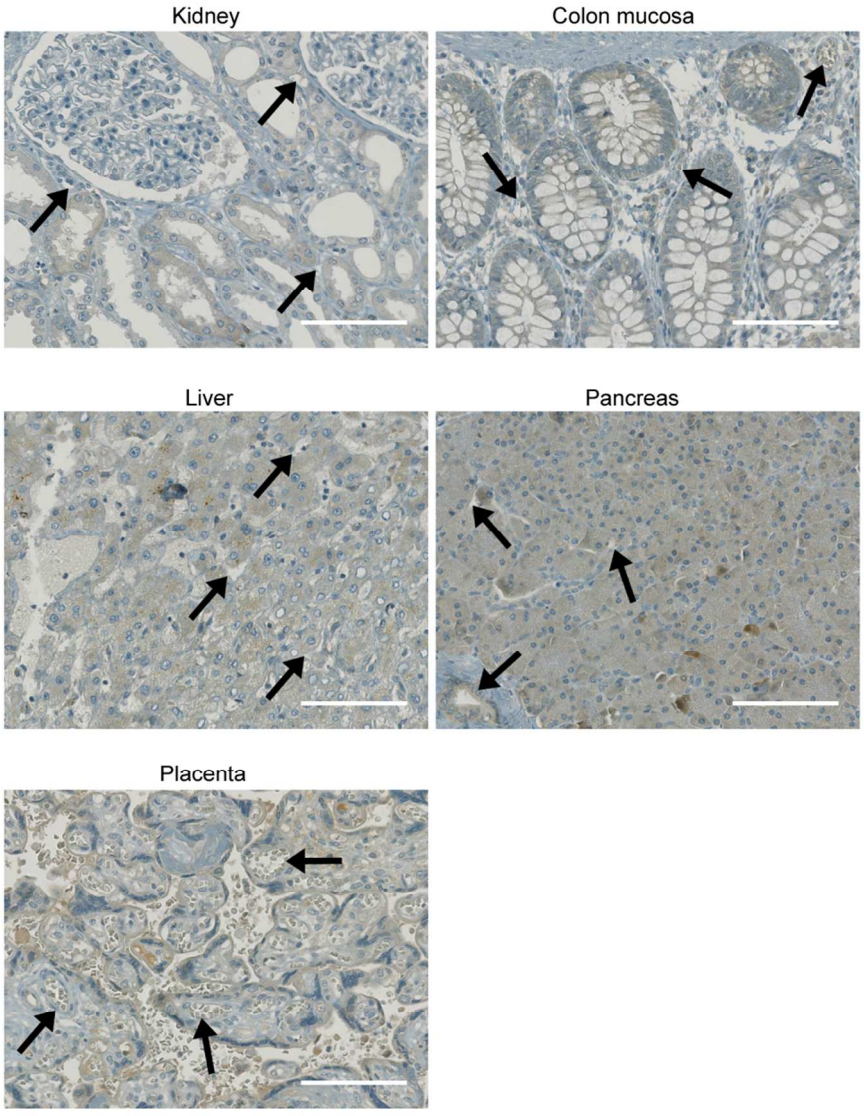
Table S2. Clinico-pathologic characteristics of the patients with non-invasive bladder cancer whose samples are contained in the TMA. Quantification of immunohistochemical staining for insulin receptor on blood vessels in the TMA

Table S3. Sequences of PCR and RT-qPCR primers

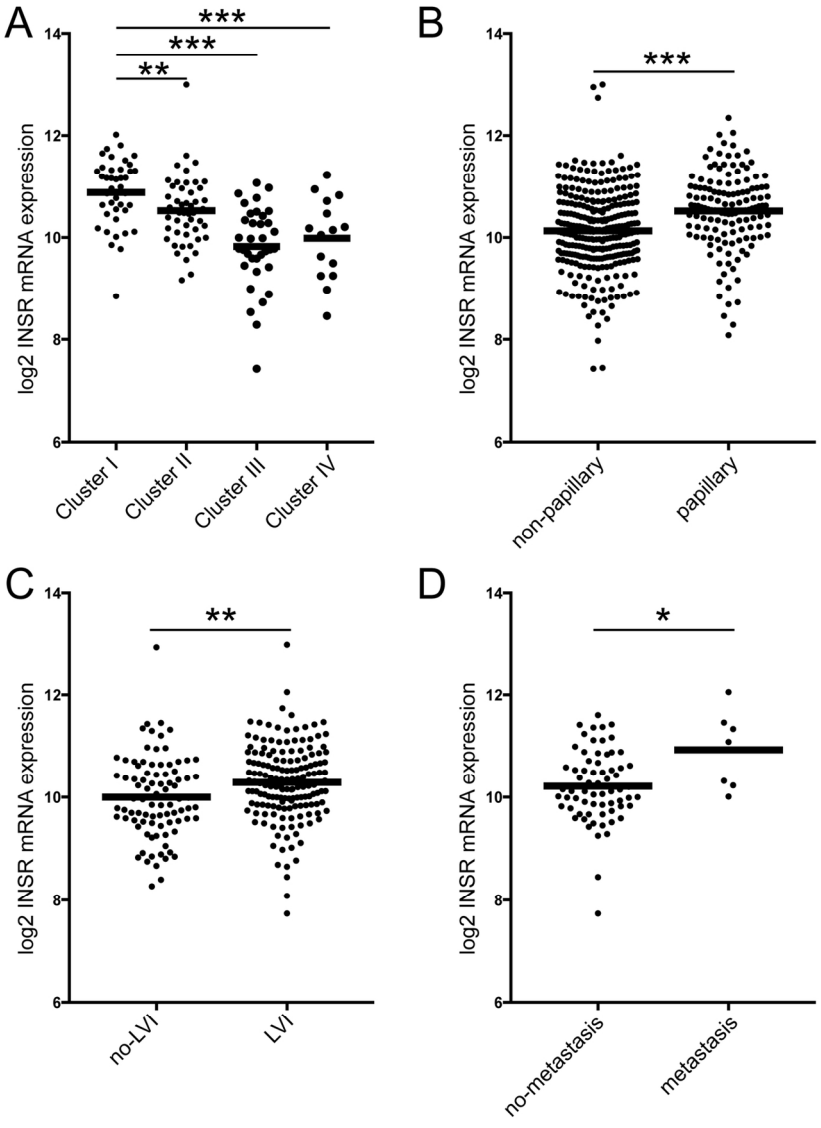
Supplementary Figure 1



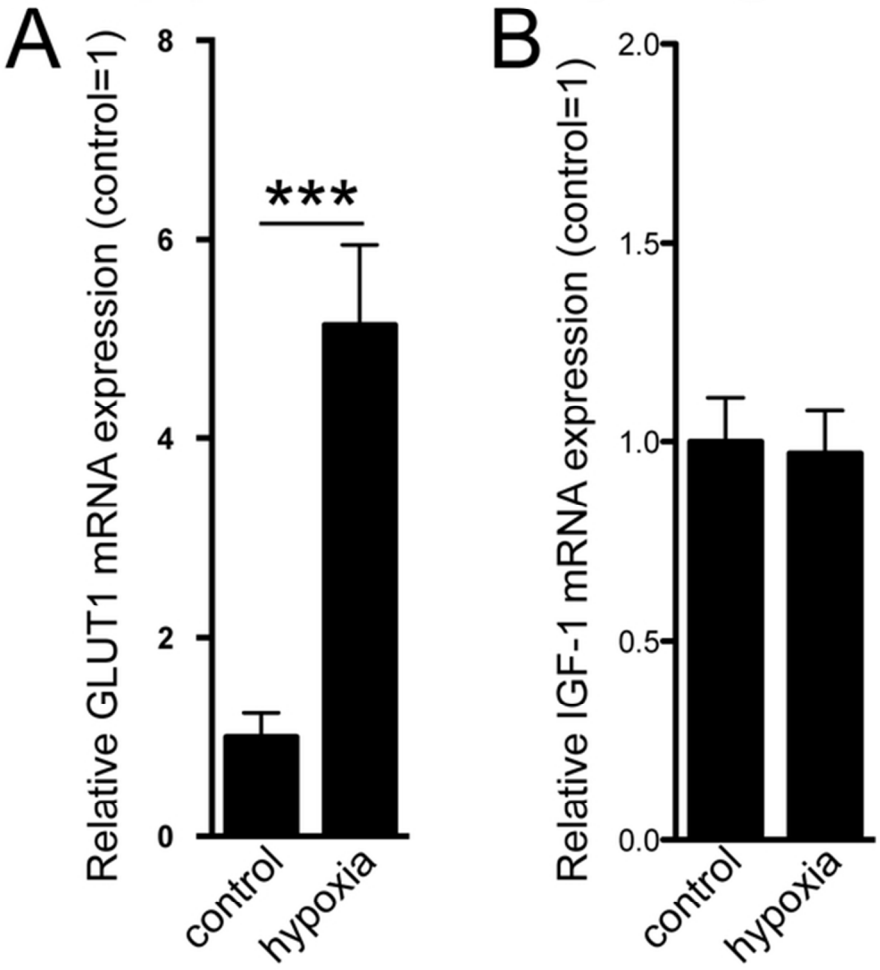
Supplementary Figure 2



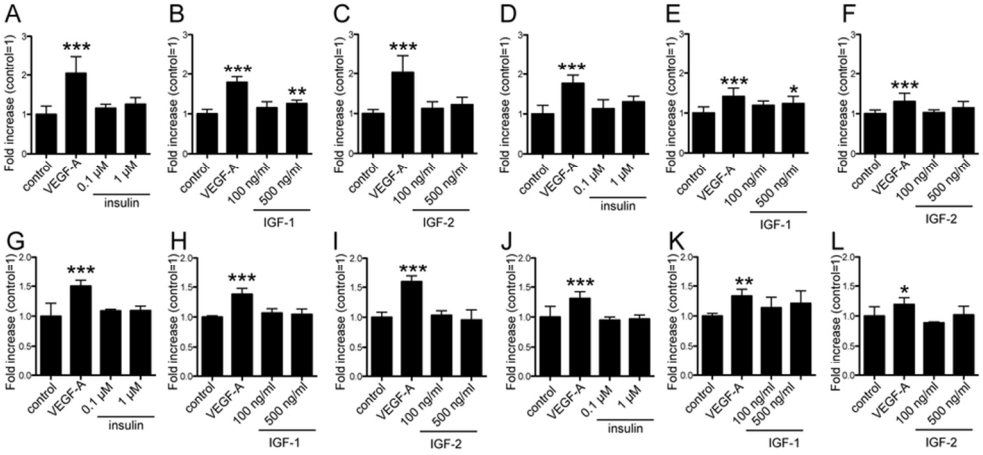
Supplementary Figure 3



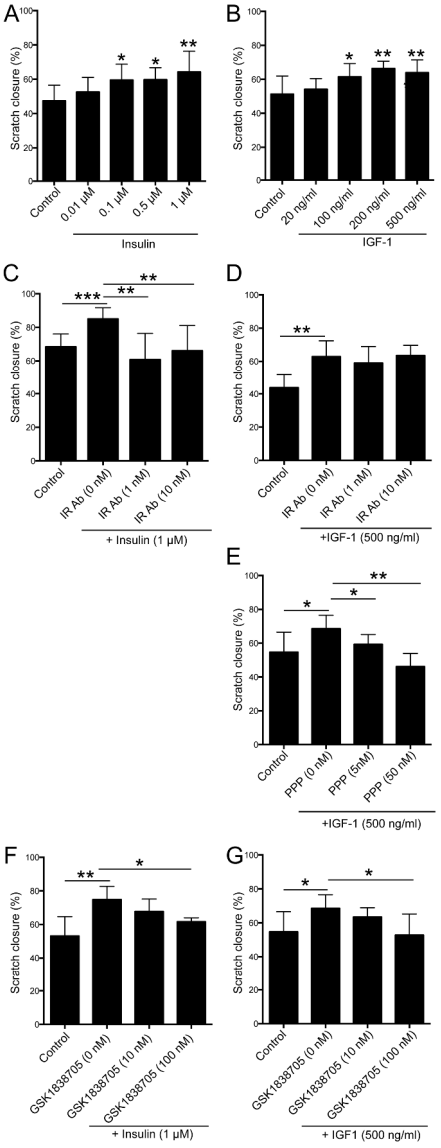
Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6



Supplementary Table S1. Clinico-pathologic characteristics of the patients with invasive bladder cancer whose samples are contained in the TMA. Quantification of immunohistochemical staining for insulin receptor on blood vessels and GLUT1 on tumor cells in the TMA.

Variable	Categorization	n analyzable	%
Total number of patients=66			
Clinico-pathologic data:			
Age at diagnosis (median, range)		63.35 (43-82)	
	<70	47	71.2
	≥70	19	28.8
Sex			
	Female	16	24.2
	Male	50	75.8
Tumor stage (WHO 1973)			
	Solitary CIS	1	1.5
	pTa	2	3.0
	pT1	9	13.6
	pT2	18	27.3
	pT3	27	40.9
	pT4	9	13.6
Histologic grade (WHO 1973)			
	G2	5	7.6
	G3	61	92.4
Adjacent carcinoma in situ			
	No	39	59.1
	Yes	27	40.9
Venous invasion			
	No	54	81.8
	Yes	12	18.2
Lymphatic invasion			
	No	48	72.7
	Yes	18	27.3
Lymph node positive			
	No	40	60.6
	Yes	26	39.4
Immunohistochemistry (IHC):			
Insulin receptor			
	Absent, weak or medium expression	51	77.3
	Strong expression	12	18.2
	Few vessels observed	3	4.5
GLUT1 (SLC2A1)			
	Absent or weak expression	30	47.6
	Strong expression	33	52.4

Supplementary Table S2. Clinico-pathologic characteristics of the patients with non-invasive bladder cancer whose samples are contained in the TMA. Quantification of immunohistochemical staining for insulin receptor on blood vessels in the TMA.

Variable	Categorization	n analyzable	%
Total number of patients=90			
Clinico-pathologic data:			
Age at diagnosis (median, range)		68.5 (32-88)	
	<70	49	54.4
	≥70	41	45.6
Sex			
	Female	20	22.2
	Male	70	77.8
Tumor stage (WHO 1973)			
	pTa	90	100.0
Histologic grade (WHO 1973)			
	G1	39	43.3
	G2	48	53.3
	G3	3	3.3
Histologic grade (WHO 2004)			
	Low grade	72	80.0
	High grade	18	20.0
Adjacent carcinoma in situ			
	No	89	98.9
	Yes	1	1.1
Multiplicity			
	Solitary	74	82.2
	Multifocal	16	17.8
Growth pattern			
	papillary	89	98.9
	solid	1	1.1
Immunohistochemistry (IHC):			
Insulin receptor			
	No or weak expression	37	41.1
	Strong expression	53	58.9

Supplementary Table S3. Sequences of PCR and RT-qPCR primers

Gene symbol	species	purpose	sequence 5'-3'
SLC2A1 (GLUT1)	human	RT-qPCR	GGCCAAGAGTGTGCTAAAGAA (forward) ACAGCGTTGATGCCAGACAG (reverse)
IGF1	human	RT-qPCR	GCAATGGGAAAAATCAGCAG (forward) GAGGAGGACATGGTGTGCA (reverse)
IGF2	human	RT-qPCR	CCGTGCTTCCGGACAACTT (forward) CTGCTTCCAGGTGTCATATTGG (reverse)
INSR	human	RT-qPCR	AGGAGCCCAATGGTCTGA (forward) GAGACGCAGAGATGCAGC (reverse)
INSR-A	human	RT-qPCR	TGGTTTTCGTCCCCAGGC (forward) CCACCGTCACATTCCCAAC (reverse)
INSR-B	human	RT-qPCR	GTGCCGAGGACCCTAGGCC (forward) CCACCGTCACATTCCCAAC (reverse)
INSR	mouse	genomic PCR (detecting flox sequence)	CTCAACTGAGATCCCCCTCA (forward) CTGAATAGCTGAGACCACAG (reverse)
INSR	mouse	RT-qPCR for detecting deletion of exon 4	ATAAGTGCATCCCCGAGTGC (forward) CTGAATAGCTGAGACCACAG (reverse)
RPLP0	human	RT-qPCR reference gene	CAGATTGGCTACCCAACTGTT (forward) GGGAAGGTGTAATCCGTCTCC (reverse)
RPLP0	mouse	RT-qPCR reference gene	AGATTCGGGATATGCTGTTGGC (forward) TCGGGTCCTAGACCAGTGTTC (reverse)